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Transcriptional regulator Sar regulates the multiple secretion systems in *Xanthomonas oryzae*

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Abstract

Xanthomonas oryzae pv. *oryzae* (Xoo) is a notorious plant pathogen that causes leaf blight of rice cultivars. The pathogenic bacteria possess numerous transcriptional regulators to regulate various biological processes, such as pathogenicity in the host plant. Our previous study identified a new master regulator PXO_RS20790 that is involved in pathogenicity for Xoo against the host rice. However, the molecular functions of PXO_RS20790 are still unclear. Here, we demonstrate that transcriptional regulator Sar (PXO_RS20790) regulates multiple secretion systems. The RNA-sequencing analysis, bacterial one-hybrid assay, and electrophoretic mobility shift assay revealed that Sar enables binding of the promoters of the T1SS-related genes, the avirulence gene, *raxX*, and positively regulates these genes' expression. Meanwhile, we found that Sar positively regulated the T6SS-1 clusters but did not regulate the T6SS-2 clusters. Furthermore, we revealed that only T6SS-2 is involved in interbacterial competition. We also indicated that Sar could bind the promoters of the T3SS regulators, *hrpG* and *hrpX*, to activate these two genes' transcription. Our findings revealed that Sar is a crucial regulator of multiple secretion systems and virulence.

KEYWORDS
RaxX, regulation, secretion system, transcriptional regulator, *Xanthomonas oryzae*

1 | INTRODUCTION

The plant pathogen *Xanthomonas oryzae* pv. *oryzae* (Xoo), a gram-negative bacterium belonging to the class gamma-proteobacteria (Timilsina et al., 2020), is an important bacterial pathogen that severely affects rice yields worldwide by causing leaf blight disease (Jiang et al., 2020). Due to its economic or scientific importance, plant bacteriologists selected *X. oryzae* pv. *oryzae* as one of the "top 10" bacterial plant pathogens (Mansfield et al., 2012). To adapt to the multiple changing environmental factors and survive in the host plant, the physiological metabolism of bacteria needs to be precisely regulated (Li et al., 2020; Yang et al., 2020; Zheng et al., 2021). Numerous virulence-associated factors facilitate bacterial proliferation in the host by suppressing the plant immunity and promoting symptom development (Buttner & Bonas, 2010; Timilsina et al., 2020), including extracellular polysaccharide (EPS) (Xue et al., 2018), lipopolysaccharide (LPS) (Petrocelli et al., 2012), secretion systems and effectors (Alvarez-Martinez et al., 2021; Timilsina et al., 2020). Among all the virulence-associated factors, the secretion systems are the most important (Alvarez-Martinez et al., 2021). In gram-negative bacteria, at least six secretion systems have been reported, from the type I secretion system to the type VI secretion

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system (T1SS to T6SS), and these secretion systems are vital to pathogenic bacterial survival in complex environments, especially in the host plant (Alvarez-Martinez et al., 2021; Costa et al., 2015).

In *Xanthomonas*, the T1SS consists of three genes, *raxA*, *raxB*, and *raxC*, which encode a membrane protein, a peptidase-containing ABC transporter, and an outer membrane protein, respectively (da Silva et al., 2004; Han et al., 2012; Liu et al., 2019). The first gene in the *raxSTAB* operon, *raxST*, encodes a sulfotransferase-like protein (Han et al., 2012). A sulfated RaxX (AvrXa21) secreted by the T1SS is required to activate Xa21-mediated recognition and immunity (Ercoli et al., 2022; Han et al., 2012; Luu et al., 2019; da Silva et al., 2004). It has been reported that the RaxR-RaxH two-component system (TCS) regulates the expression of eight *rax* genes (Lee et al., 2006, 2008). Meanwhile, the PhoP-PhoQ TCS is negatively regulated by the RaxR-RaxH TCS and is also required for AvrXa21 activity (Lee et al., 2008). A study revealed a key T3SS-related response regulator HrpX directly regulating the expression of *raxSTAB-raxX*; moreover, the HrpX-binding plant-inducible promoter (PIP) box was found in the promoter region of *raxSTAB* and *raxX*, respectively (Joe et al., 2021). It is worth noting that our previous RNA-sequencing (RNA-Seq) data showed that some pathogenicity-related regulators were participating in *raxSTAB* and/or *raxX* expression, such as HrpG, HrpX, VemR, and PXO_RS20790 (Zheng et al., 2021). However, the regulation of *raxX* and *raxSTAB* cluster expressions remains elusive.

The T6SS is a bacterial contact-dependent contractile nanomachine used to inject proteinaceous protein effectors into eukaryotic or prokaryotic cells to manipulate the host and direct killing of competitors in complex communities (Wang et al., 2019; Yu et al., 2021). In *Pseudomonas aeruginosa*, the T6SS has displayed a significant role in interspecies competition, transportation of metal ions, and diverse stresses (Basler et al., 2013; Han et al., 2019; Hood et al., 2010; Lin et al., 2015, 2017). In plant pathogens such as *Xanthomonas citri*, the T6SS was required to resist *Dictyostelium discoideum* predation (Bayer-Santos et al., 2018). In addition, the T6SS of *Xanthomonas* species is also involved in pathogenicity (Montenegro Benavides et al., 2021). Some *Xanthomonas* strains have two phylogenetically distinct T6SS clusters, T6SS-1 and T6SS-2 (Bayer-Santos et al., 2019). A recent study reported that T6SS-2 plays a vital role in interspecies competition in *X. oryzae* pv. *oryzicola* (Zhu et al., 2020). However, the biological functions and regulations of T6SSs have not been elucidated in most *Xanthomonas* species.

Among all these secretion systems, T3SS plays a crucial role in both animal and plant pathogens of successful infection in the host (Deng et al., 2017; McCann & Guttman, 2008). T3SS is encoded by the *hrp* gene clusters, containing multiple transcription elements consisting of more than 20 genes (Buttner & Bonas, 2002). In *X. oryzae*, the activation of *hrp* gene clusters in plants or on the basic medium XOM2 (*X. oryzae* pv. *oryzae hrp*-inducing medium 2) requires transcriptional regulators (TRs) HrpG and HrpX (Tsuge et al., 2002). They regulate T3SS by regulating the expression of *hrp* gene clusters (Guo et al., 2011). HrpG is an OmpR family response regulator that belongs to the two-component system and activates the expression of *hrpX*, which encodes an AraC-type regulator (Teper et al., 2021). HrpX binds to the PIP box region upstream of the T3SS gene clusters to regulate the expression of T3SS-related genes (Koebnik et al., 2006). Although a few studies have been reported to identify regulators that could directly activate *hrpG* and/or *hrpX* transcription, many TRs have not been identified (An et al., 2011; Huang et al., 2009).

The GntR TR family is a broad and abundant TR family in prokaryotes (Hoskisson & Rigali, 2009). Among bacteria, members of the GntR regulate various biological processes, including oxidative stress, metabolic pathways, motility and adhesion, and pathogenicity (Su et al., 2016; Zhou et al., 2017). However, many TRs in bacterial species belonging to the genus *Xanthomonas* have never been investigated. In the present study, we report that a versatile TR Sar regulates the expression of T1SS, T3SS, and T6SS.

2 | RESULTS

2.1 | *PXO_RS20790* of *X. oryzae* is involved in oxidative stress

Our previous study identified a novel TR PXO_RS20790, a new master pathogenicity-associated regulator in almost all regulators in the PXO99^A genome (Zheng et al., 2021) (Figure S1a,b). The *PXO_RS20790* gene (named *sar* in this study) is annotated to encode a GntR-family TR with an N-terminal DNA-binding domain and a C-terminal UTRA domain (Figure 1a). Several pathogenicity-related phenotypes were investigated to explore the factors that resulted in the virulence attenuation of the *PXO_RS20790* deletion mutant. First, in vitro growth assays were performed for comparisons between wild-type and *PXO_RS20790* mutant strains. The results showed that no significant difference was found between *PXO_RS20790* mutant and WT strain growth in nutrient broth (NB) or XOM2 medium (Figure 1b,c). In addition, the results also showed that the *PXO_RS20790* mutant had no significant influence on other pathogenicity-related phenotypes, including exopolysaccharides (EPS) (Dharmapuri & Sonti, 1999; Kim et al., 2009), swarming motility (Qi et al., 2020), and extracellular cellulase (Tayi et al., 2018) (Figure S1c). However, the *PXO_RS20790* deletion mutant was more sensitive to hydrogen peroxide (H_2O_2), which, as one of the reactive oxygen species (ROS), compared with that in wild-type strain PXO99^A, and a complemented strain showed that H_2O_2 resistance could be restored to the wild type (Figure 1d). These results indicate that *PXO_RS20790* is involved in the pathogenicity and oxidative stress of *X. oryzae* pv. *oryzae*.

2.2 | Detecting PXO_RS20790 regulons by bacterial one-hybrid

Our previous RNA-Seq analysis revealed that 428 genes were differentially expressed in the PXO_RS20790 mutant compared with those in PXO99^A (Zheng et al., 2021). These observations showed that PXO_RS20790 directly or indirectly regulates expression of numerous genes, especially secretion system-associated genes.

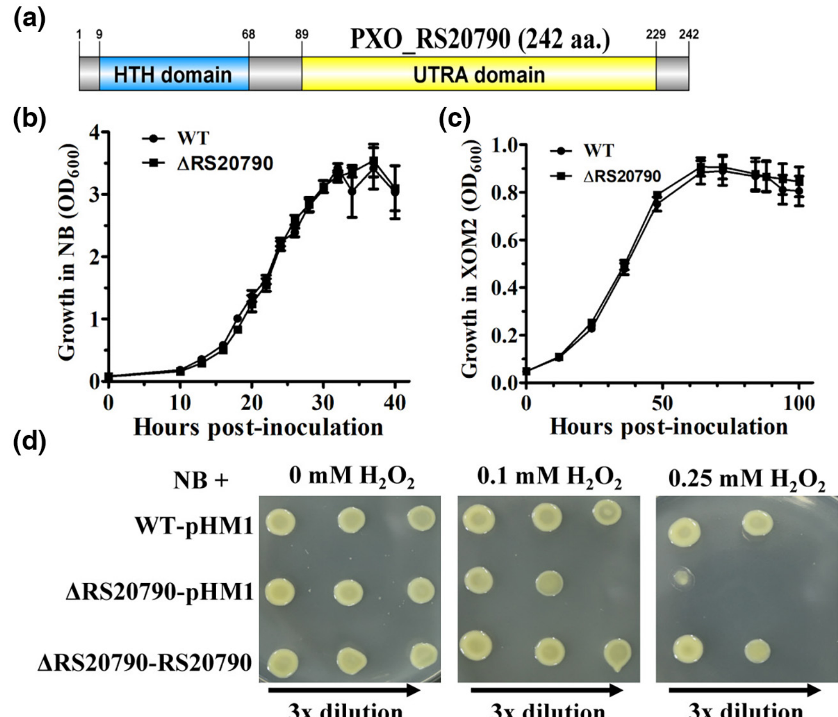

FIGURE 1 The PXO_RS20790 protein participated in oxidative stress. (a) Schematic view of the putative secondary structures of PXO_RS20790 according to the Pfam database. HTH, helix-turn-helix domain; UTRA, UbiC transcription regulator-associated. (b,c) *Xanthomonas oryzae* pv. *oryzae* (Xoo) wild-type (WT) or mutant (ΔRS20790) strains were cultured in 5 ml of nutrient broth (NB) for 18–24 h to the middle logarithmic phase. Xoo cells were then harvested, washed using sterile water, and finally adjusted to an OD_{600} of 1.5. The resuspended Xoo culture was inoculated to NB medium (b) or XOM2 medium (c) by 2%. The optical density (OD) at 600 nm of the cultures was measured, and values are computed as the mean ± standard deviation of three technical replicates. WT, wild type. (d) The hydrogen peroxide (H_2O_2) sensitivity of Xoo strains. Bacterial cultures (OD_{600} = 0.8) were pipetted onto NB agar without or with 0.1 mM and 0.25 mM H_2O_2. The plates were kept for 48–72 h at 28°C. All the experiments were repeated at least three times, and one representative is shown

To further identify genes or operons regulated directly by PXO_RS20790 in Xoo PXO99[A], we identified PXO_RS20790-binding promoters by the bacterial one-hybrid system. We constructed a promoter sublibrary containing 396 operons that contain the PXO_RS20790-regulated operons. The full length of PXO_RS20790 was used as bait to screen the promoters in these 396 operons. The results showed that PXO_RS20790 interacted with 74 promoters (Figure S2 and Table S3). The 74 targets could be divided into 12 classes according to the function of these genes (Figure 2a). It is noteworthy that PXO_RS20790 interacted with the promoter of secretion system-related genes, including T1SS, T3SS, and T6SS (Figure 2a).

Based on the bacterial one-hybrid results, a consensus PXO_RS20790-binding DNA motif (WSKKCMDGCGCHRSN) was predicted from the putative promoter regions of the 74 Sar-regulated operons (Figure 2b). Furthermore, we employed an electrophoretic mobility shift assay (EMSA) to verify this interaction. The EMSA verified physical binding between PXO_RS20790 and a synthetic double-stranded DNA consensus motif (WSKKCMDGCGCHRSN). The binding almost disappeared when this DNA motif's nucleotides were mutated (Figure 2c). Next, a synthetic double-stranded DNA motif GGCGTCGAGCGCCAGGC was examined based on the conserved motif of PXO_RS20790. We found that PXO_RS20790 interacted with this motif but could not bind the mutated motif (Figure S3a). Taken together, these findings demonstrated that PXO_RS20790 acts as a TR for the expression of secretion systems. Therefore, PXO_RS20790 was named Sar, which stands for the secretion-associated TR.

2.3 | Sar positively regulates the expression of T1SS-related genes and *raxX*

The rice receptor-like kinase Xa21 provides immunity against Xoo strains that carry *raxX* (*AvrXa21*). The T1SS-related *rax* genes of Xoo are also required for RaxX activity (Ercoli et al., 2022). Sar could be involved in activating the transcription of the *raxSTAB* gene cluster according to our previous RNA-Seq data and the bacterial one-hybrid assay (Zheng et al., 2021) (Figure 2a). A previous study showed that *raxST*, *raxA*, and *raxB* probably constitute an operon (da Silva et al., 2004). Reverse transcription PCR (RT)-PCR results also indicated that *raxST*, *raxA*, and *raxB* are co-transcribed (Figure S4). First, a quantitative real-time reverse transcription PCR (RT-qPCR) assay was performed to determine whether Sar has any regulatory relations with the T1SS *rax* cluster (Figure 3a). The analysis indicated that *sar* deletion resulted

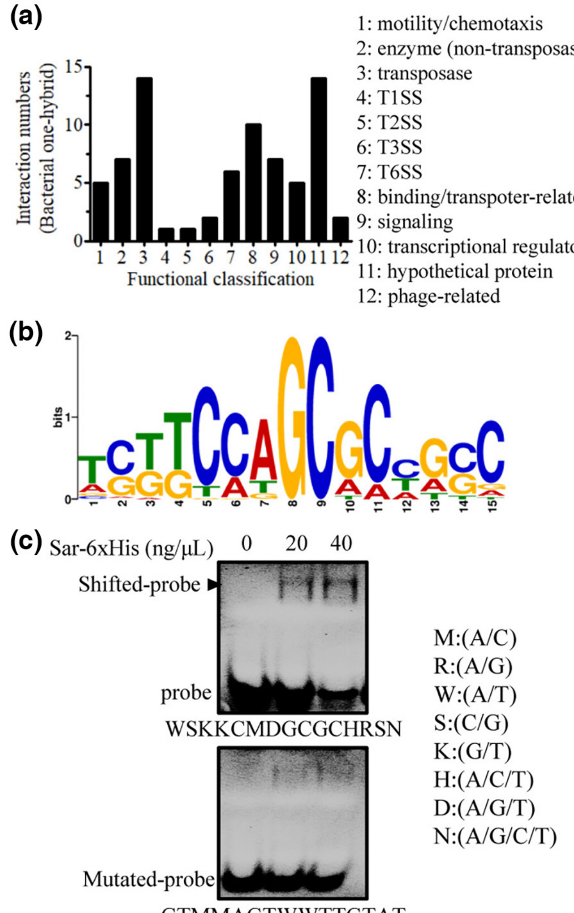

FIGURE 2 The analysis of the PXO_RS20790 regulon by bacterial one-hybrid assay. (a) Functional categories of the genes with promoters that were putatively bound by PXO_RS20790. Details of the genes are given in Table S3. (b) Predicted consensus PXO_RS20790-binding DNA motifs based on bacterial one-hybrid data and MEME suite analysis. (c) Verification of PXO_RS20790-binding DNA motif by electrophoretic mobility shift assay (EMSA). Two oligonucleotides were synthesized and incubated with the PXO_RS20790 protein in the EMSA, which was used to measure the interaction between the PXO_RS20790 protein and the oligonucleotide wild-type (top) and mutated (bottom) DNA probes according to the conserved Sar binding motifs in (b). Similar results were observed in two independent experiments

in the down-regulation of *raxST*, *raxA*, *raxB*, and *raxX*, suggesting that Sar positively activates the expression of the *raxSTAB* operon and avirulence gene *raxX* (Figure 3b). Second, we constructed a recombinant pHG3 vector to verify *raxST* and *raxX* promoter activities in WT and *sar* mutant strains, respectively. Quantitative β-glucuronidase (GUS) assays showed that both *raxST* and *raxX* promoter GUS activities of the *sar* mutant were significantly lower compared with those of WT strain PXO99^{A} (Figure 3c). Moreover, the bacterial one-hybrid assay confirmed that the TR Sar interacted with the *raxST* promoter (Figure S2e 5–10 and Table S3). The promoter regions of *raxST* and *raxX* were amplified and used as DNA probes in an EMSA to examine whether Sar directly regulates *rax* gene expression. The shifted 6-carboxy-fluorescein (FAM)-labelled DNA bands were observed when *raxST* or *raxX* promoter DNA was incubated with increasing amounts of Sar protein, and the shifted DNA progressively disappeared when the nonlabelled DNA was added to the competitive assay (Figure 3d,e). The Sar binding motif was found in *raxST* and *raxX* promoter regions according to the EMSA (Figure S3b). Deletion of the Sar-binding motif in the *raxST* promoter region significantly reduced the mRNA level of *raxSTAB* compared to the WT strain (Figure 3f). The results confirmed that Sar could control *rax*-related gene transcription by binding to these two promoters.

To further verify Sar positively regulated RaxX protein expression, the 6×His C-terminal tag was knocked in *the raxX* gene in the WT strain and *sar* mutant strain. These two strains were cultured in NB and XOM2 medium. The western blotting assay showed that *raxX* translated proteins. The expression of RaxX was decreased in the Δ*sar* mutant in *hrp*-inducing XOM2 medium compared with that in the WT strain (Figure 3g). Taken together, the results indicate that Sar binds to the promoter region of T1SS and *raxX* directly and positively controls T1SS and *raxX* expression.

2.4 | Sar regulates the expression of T6SS-1 related genes but not the T6SS-2

Gram-negative pathogens have a multifunctional type VI secretion system (T6SS), affecting interbacterial competition, virulence to the eukaryotic host, and metal ions uptake (Bernal et al., 2018; Lin et al., 2017; Sana et al., 2016). There are two phylogenetically distinct T6SS gene clusters, T6SS-1 and T6SS-2, in the genome of Xoo PXO99^{A}. However, the function and regulation of T6SSs in *Xanthomonas* species are poorly understood. According to the bacterial one-hybrid results, Sar could bind the six promoter regions of T6SS operons (Figures 2a and 4a, and Table S3). We found that T6SS-1 gene clusters were activated on the plant-mimicking XOM2 medium; in contrast, T6SS-2 related gene expression was induced under nutrient-rich conditions (NB medium) (Figure S5a,b). Therefore, we deduced that T6SS-1 and T6SS-2 have different functions.

Subsequently, the RT-qPCR assay was performed to verify the regulation relationship between Sar and T6SS-related genes. The WT and *sar* mutant strains were cultivated in XOM2 medium, and the mRNA levels of *PXO_00245*, *PXO_00263*, *PXO_00264* (*hcp1*), and *PXO_00265* in the *sar* mutant were significantly decreased compared with those in the WT strain (Figure 4b). Next, we measured the T6SS-2-related gene expression of Xoo strains in NB medium. The mRNA levels of *PXO_02045*, *PXO_02046*, and *PXO_02047* (*hcp2*) in the *sar* mutant were increased slightly compared to those in the WT strain (Figure 4c). Thus, Sar may regulate T6SS-1-related gene expression, but not T6SS-2. The promoter regions of *PXO_00244* and *PXO_00266* were amplified and used as

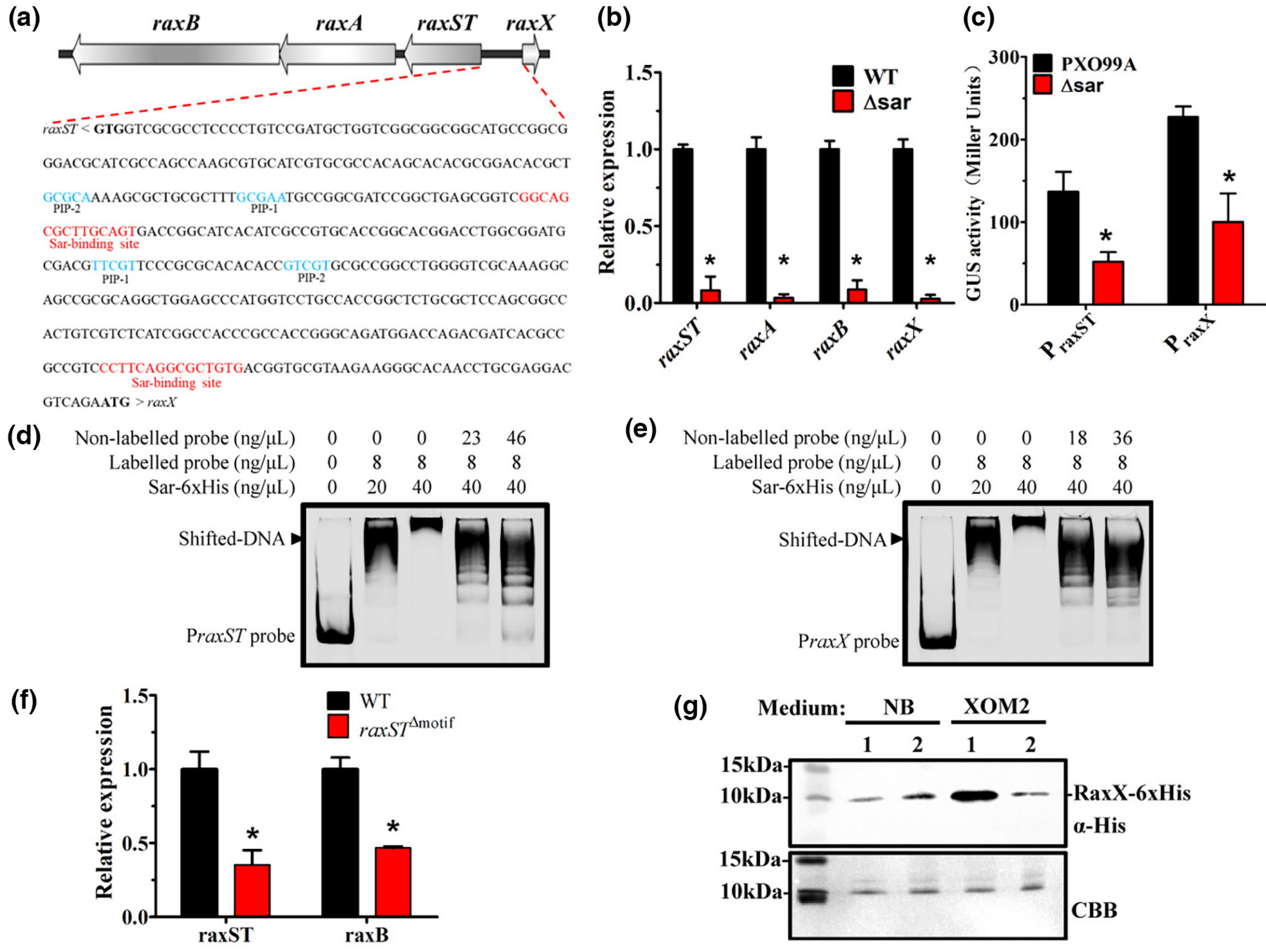

FIGURE 3 Sar regulates the expression of type I secretion system (T1SS)-related and *raxX* genes by directly binding to their promoters. (a) Schematic view of the genomic organization of the *rax*-related gene cluster. PIP-1/2 indicates the HrpX binding site in the promoters of *raxST* and *raxX*. (b) Sar positively controls the expression of *raxST*, *raxA*, *raxB*, and *raxX*. Relative expression of *rax*-related genes (*raxST*, *raxA*, *raxB*, and *raxX*) in the wild-type (WT) strain and *Sar* mutant strain Δ*sar* cultured in XOM2 medium determined by reverse transcription-quantitative polymerase chain reactions (RT-qPCRs). The cDNA of 16S rRNA was used as an internal control. Error bars indicate the standard deviation of three technical replicates. (c) The *raxST* and *raxX* promoter β-glucuronidase (GUS) activities of the *Xanthomonas oryzae* pv. *oryzae* (Xoo) WT PXO99^{A} and the *Sar* mutant Δ*sar*. Error bars indicate the standard deviation of three technical replicates. (d,e) The direct interaction between Sar protein and *raxST* and *raxX* promoters DNA tagged with 6-carboxy-fluorescein (FAM) was detected by electrophoretic mobility shift assay (EMSA). The final concentrations of Sar and DNA in the 20-μl reaction system are shown above each lane. Shifted bands indicate the probe DNA that interacts with the Sar protein. The unlabelled promoter DNA was added to the incubation system to compete with the interaction between Sar and the FAM-labelled probe. (f) The mutation of the Sar-binding site in *raxST* promoter significantly reduced the mRNA level of *raxST* and *raxB* genes by RT-qPCR. Error bars indicate the standard deviation of three technical replicates. (g) RaxX quantitative analysis at the posttranscriptional level by western blot analysis. Relative expression of RaxX in Xoo strains cultured in nutrient broth (NB) and XOM2 medium indicated by the intensity of the western blot band. The result of Coomassie brilliant blue (CBB) staining on the lower panel shows that a similar amount of bacterial total protein was loaded on each lane. Western blot and CBB staining were under the same experimental conditions for protein sample preparation and SDS-PAGE. Lane 1, *raxX*::6×His/WT; lane 2, *raxX*::6×His/Δ*sar*. In b, c, and f, asterisks indicate significant differences compared with the WT strain (mean ± *SD*, $n = 3$, $*p < 0.05$). Similar results were observed in two independent experiments

FAM-labelled DNA in an EMSA to examine whether Sar directly regulates transcription of T6SS-1 operons. The results showed that the Sar protein bound to these two promoters (Figure 4d). Therefore, the Sar protein regulates T6SS-1 expression but does not regulate T6SS-2.

Hcp protein is a vital component of the T6SS tube that is required for the secretion of T6SS effectors (Silverman et al., 2013). Therefore, *hcp* deletion mutants were obtained in Xoo PXO99^{A} and named Δ*hcp1* (the mutant in T6SS-1), Δ*hcp2* (the mutant in T6SS-2), and Δ*hcp1*Δ*hcp2*. Plant inoculation revealed that neither *hcp1* nor *hcp2* was required for the full virulence of the Xoo PXO99^{A} strain (Figure S5c).

An antagonism assay was performed to identify whether T6SS-1 or T6SS-2 participated in interbacterial killing (Silverman et al., 2013). To determine if the Xoo strains (WT, Δ*sar*, Δ*hcp1*, Δ*hcp2*, and Δ*hcp1*Δ*hcp2*) can target other bacteria, we tested them

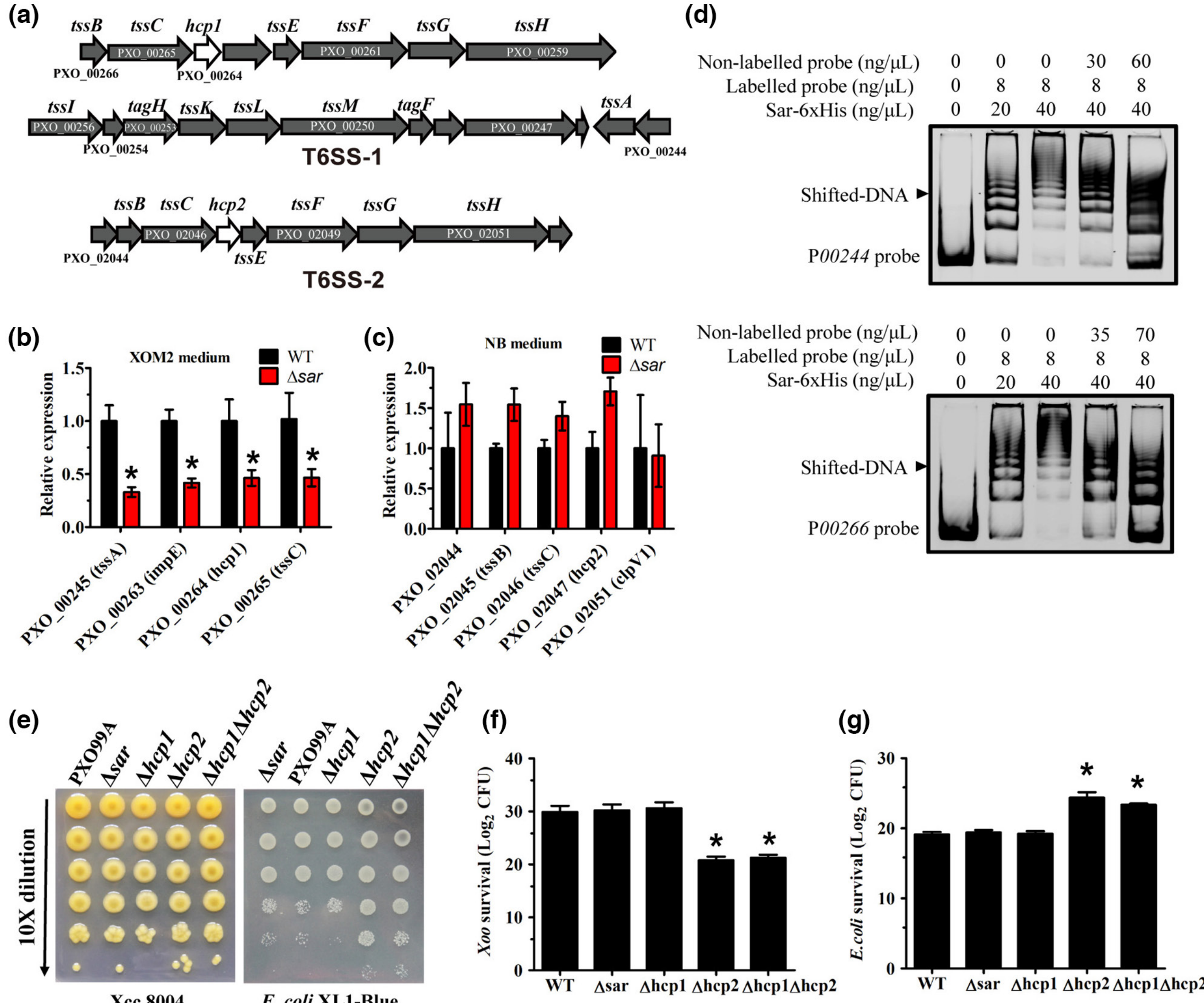

FIGURE 4 Sar is not required for interbacterial competition. (a) The genomic organization of the T6SS-1 and T6SS-2 gene clusters, which are probably regulated by Sar in PXO99^{A}. (b,c) Relative expression of T6SS-related genes in PXO99^{A} and Δ*sar* detected by reverse transcription-quantitative PCR. Significance was tested by Student's *t* test (*$p < 0.05$). Error bars indicate the standard deviation of three technical replicates. (d) The direct interaction between the Sar protein and *PXO_00244* and *PXO_00266* promoter DNA tagged with 6-carboxy-fluorescein (FAM) was detected by electrophoretic mobility shift assay (EMSA). The final concentrations of Sar and DNA in the 20-μl reaction system are shown above each lane. Shifted bands indicate the probe DNA interacts with the Sar protein. The unlabelled promoter DNA was added to the incubation system to compete with the interaction between Sar and the FAM-labelled probe. P*00244* and P*00266* are the abbreviations for the promoter DNA of *PXO_00244* and *PXO_00266*, respectively. (e) Hcp2 (T6SS-2) is vital to the competition for Xoo PXO99^{A}. Different *Xanthomonas oryzae* pv. *oryzae* (Xoo) cells compete with *Xanthomonas campestris* pv. *campestris* (Xcc) 8004 and *Escherichia coli* XL1-Blue. The images show Xcc 8004 (left) and *E. coli* XL1-Blue (right) with a serial 10-fold dilution spotted on nutrient broth (NB) agar with rifampicin plates and Luria-Bertani (LB) agar with kanamycin plates, respectively. (f,g) The bacterial CFU for Xoo strains (f) and *E. coli* (g). Xoo strains possess a pHM1 cosmid (spectinomycin resistance) and the *E. coli* XL1-Blue strain has a kanamycin resistance gene. The different Xoo–*E. coli* strains were co-cultured strains with serial 10-fold dilutions. Then, the diluted samples were spread on spectinomycin NB agar plates and kanamycin LB agar plates, respectively. The bacterial populations of Xoo (f) and *E. coli* (g) were counted. Similar results were observed in two independent experiments

with two different strains of *Xanthomonas campestris* pv. *campestris* 8004 (Xcc 8004) and *Escherichia coli* XL1-Blue for interspecies and intergeneric competition, respectively. Our results showed that the Δ*hcp1* (T6SS-1) of Xoo PXO99^{A} and the Δ*sar* strain did not antagonize these Xcc or *E. coli*; however, the Δ*hcp2* (T6SS-2) did not antagonize Xcc 8004 but did antagonize *E. coli* (Figure 4e). The bacterial CFU assay was also performed between Xoo strains and the competitor *E. coli*. The results revealed that the bacterial population of Δ*hcp2* was significantly reduced compared with that of the WT strain, but this mutant strain could promote competitor *E. coli* survival (Figure 4f,g). The results indicate that the T6SS-2 in Xoo PXO99^{A} is involved in competition with *E. coli*, consistent with the recent study in *X. oryzae* pv. *oryzicola* (Zhu et al., 2020). Taken together, these results demonstrate that Sar positively regulates T6SS-1 gene expression, but does not regulate T6SS-2 gene expression.

2.5 | Sar is involved in activation of the expression of T3SS through HrpG and HrpX

In *X. campestris* pv. *vesicatoria*, HrpX has been shown to regulate *hrp* gene transcription by directly binding to the PIP box in the promoters of *hrp* operons (Koebnik et al., 2006). From the transcriptome of Sar, it was found that Sar positively regulates *hrp-hrc-hpa* related genes (Zheng et al., 2021). The RT-qPCR assay confirmed the RNA-Seq data. The result showed that the mRNA levels of *hrpG*, *hrpX*, and T3SS-related genes, including *sctR*, *hrcS*, and *hpaB* in the Δ*sar* mutant strain, were significantly lower than that of the WT strain (Figure 5a). These results indicate that Sar promotes the transcription of *hrpG* and *hrpX*.

The bacterial one-hybrid showed that Sar could physically interact with the promoter regions of *hrpG* and *hrpX* (Figure S2e 5–3, 5–4 and Table S3). To confirm whether Sar regulates *hrpG* and *hrpX* transcription directly, the promoter regions of *hrpG* and *hrpX* were amplified and used as DNA probes in the EMSA assay. As shown in Figure 5b, the shifted FAM-labelled DNA bands were observed when *hrpG* or *hrpX* promoter DNA was incubated with increasing amounts of Sar protein, and the shifted DNA progressively disappeared when the nonlabelled DNA was added to the competitive assay. These results indicate that Sar specifically interacts with *hrpG* and *hrpX* promoters, and directly controls the transcription of these two genes.

A recent study showed that the HrpG-binding motif is [ATT(C/T)(C/T)(G/C/A)(T/A)T] in *Xcc* 8004 (Zhang et al., 2020). The HrpG protein of Xoo PXO99^{A} shared 77% identity with HrpG$_{Xcc}$. We found the HrpG-binding motif (ATTCCATT) in the promoter region of *hrpX* (Figure 5c). We also found the putative Sar-binding site in the promoter regions of *hrpG* and *hrpX* (Figure 5c). Meanwhile, the EMSA confirmed that Sar could bind these two DNA motifs (Figure S3c). Mutation of the Sar-binding motif in the *hrpX* promoter significantly reduced the mRNA level of *hrpX* and T3SS-related genes compared to the wild-type strain (Figure 5d). Taken together, Sar could activate both *hrpG* and *hrpX* transcription.

3 | DISCUSSION

TRs are vital to bacteria survival when they encounter biotic or abiotic stresses. For pathogenic *X. oryzae*, some TRs are required for regulating virulence-related gene expression (Zheng et al., 2021). In this study, we identified the GntR-family TR Sar as a multiple secretion systems regulator in *Xanthomonas*. We verified that deletion of *sar* in Xoo resulted in the reduction of type I secretion and type III secretion gene expression. We also showed that Sar positively regulates T6SS-1, but does not regulate T6SS-2 gene expression, and T6SS-2 is vital to intergeneric competition for Xoo. Further analysis discovered that Sar directly promotes secretion system-associated gene expression. Our results revealed that Sar is a master pathogenicity-associated regulator regulating T1SS, T3SS, and T6SS associated gene expression (Figure 6).

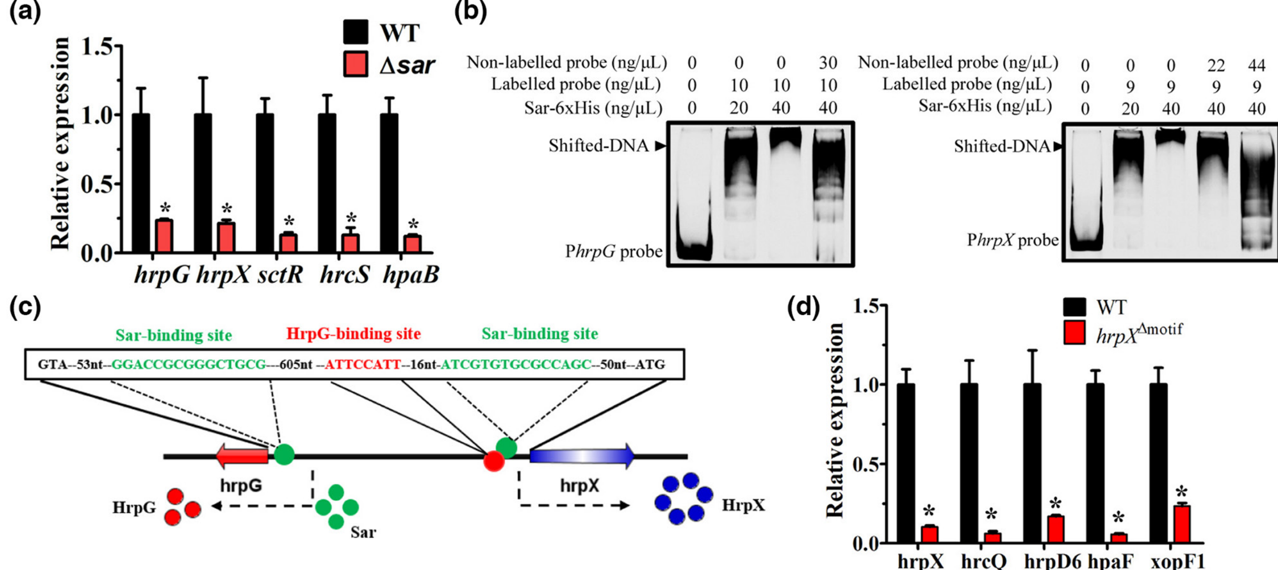

FIGURE 5 Sar positively regulated *hrpG* and *hrpX* expression. (a) Relative expression of T3SS-related genes in PXO99^{A} (WT) and Δ*sar* detected by reverse transcription-quantitative PCR (RT-qPCR). Error bars indicate the standard deviation of three technical replicates. (b) The direct interaction between the Sar protein and *hrpG*/*hrpX* promoter DNA tagged with 6-carboxy-fluorescein (FAM) was detected by electrophoretic mobility shift assay (EMSA). Shifted bands indicate the probe DNA interacts with the Sar protein. The unlabelled promoter DNA was added to the incubation system to compete with the interaction between Sar and the FAM-labelled probe. (c) The putative Sar-binding motifs in the promoter region of *hrpG* and *hrpX*. (d) The mutation of the Sar-binding site in the *hrpX* promoter significantly decreased the mRNA level of *hrpX* and T3SS-related genes by RT-qPCR. Error bars indicate the standard deviation of three technical replicates. In (c) and (d) significance was tested by Student's *t* test (*indicates significance at $p < 0.05$). Similar results were observed in two independent experiments

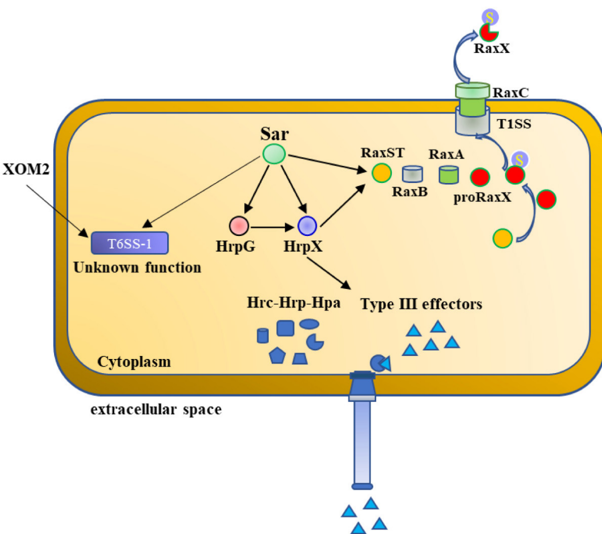

FIGURE 6 Schematic representation of the genetic regulation between Sar and its target genes in *Xanthomonas oryzae* pv. *oryzae*. Arrows indicate that a protein promotes the target. Sar involves in activation of the expression of HrpG, HrpX, RaxSTAB-RaxX, and T6SS-1. The minimal XOM2 medium can activate the transcription of T6SS-1 related genes

In *X. oryzae*, the avirulence protein RaxX is sulfated by RaxST and exported through T1SS composed of RaxA, RaxB, and RaxC. The sulfated RaxX is required for the XA21-dependent immune response (Luu et al., 2019; Pruitt et al., 2015). The production of RaxX sulfopeptide has required the transcription of the *raxST* gene. Both PhoPQ and RaxRH TCSs are required for *raxST* expression, and the T3SS regulator HrpX is a crucial regulator required for *raxST* and *raxX* expression (Burdman et al., 2004; Joe et al., 2021; Lee et al., 2008). HrpX is a positive key regulator that can bind the PIP box of *raxST* and *raxX* for transcription directly (Joe et al., 2021). Although HrpX regulates the RaxX biosynthetic pathway, the regulators that control *raxSTAB-raxX* gene expression are still elusive. We found that *raxST*, *raxA*, and *raxB* are cotranscribed in the same operon (Figure S4). We also found that the transcription levels of *raxSTAB-raxX* were significantly decreased in the *sar* mutant compared to that of the wild-type strain (Figure 3b,c). Further analysis showed that Sar could bind the promoter region of *raxST* and *raxX* (Figure 3d,e). We found that Sar is a positive TR located upstream of HrpX for its transcription. The expression of *raxSTAB* and *raxX* is required for both Sar and HrpX according to our previous RNA-Seq data (Figures 2 and 5) (Zheng et al., 2021). We believe that Sar is one of the switches for regulating the expression of *rax*-related genes, but not the most crucial one. HrpX is the critical regulator that controls *rax*-related gene transcription (Joe et al., 2021). Taken together, we found a novel TR that could bind to the promoter region of *raxST* and *raxX* for their expression. However, the regulation of *rax*-related genes is complicated, therefore future work should focus on the regulatory network between regulators and *rax*-related genes.

Gram-negative bacteria utilize the T6SS as a versatile weapon for survival in many competitions, such as interbacterial killing, eukaryotic toxicity, and metal ion uptake (Bayer-Santos et al., 2018; Yu et al., 2021). There are two T6SS clusters in the genome of Xoo PXO99^{A}, named T6SS-1 and T6SS-2. Our bacterial one-hybrid data showed that Sar could interact with six predicted promoters in the T6SS-1 and T6SS-2 clusters (Figure 2a and Table S3). Further analysis confirmed this interaction (Figure 4c). We found that Sar positively regulates T6SS-1, but does not regulate T6SS-2 (Figure 4a,b). Hcp is an essential component of the T6SS tube and chaperon for effectors' secretion. Neither Hcp1 nor Hcp2 were required for the pathogenicity for Xoo against host rice cultivar IR24 (Figure S5c). We also found that T6SS-2 (Hcp2), but not T6SS-1 (Hcp1), was required for competition with *E. coli* (Figure 4d), which is consistent with a recent report in *X. oryzae* pv. *oryzicola* (Zhu et al., 2020). Moreover, the *sar* and *hcp1* mutant strains showed similar phenotypes according to interspecies and intergeneric competition assays (Figure 4e–g). The results showed that Sar is not responsible for interbacterial competition like T6SS-1. More experiments should be performed to explore the Sar regulating T6SS-1 related phenotype except for interbacterial competition. The present experimental design could not result in a different phenotype due to the weak differential expression of the T6SS-2 (less than 2-fold) between the Sar mutant and the WT. An experiment with higher resolution should be designed to identify the difference regarding interbacterial competition, for example exploring the interbacterial competition ability in the infection process.

The T3SS is vital to gram-negative bacterial pathogens. In *Xanthomonas* bacteria, the virulence relies on the secretion and translocation of effectors of the T3SS controlled by two master TRs, HrpG and HrpX (Teper et al., 2021). Disruption of these two regulators in *Xanthomonas* spp. completely abolishes pathogenicity on the host plants (Kamdar et al., 1993; Wengelnik et al., 1996). Many studies focus on the regulatory networks that regulate and are regulated by the HrpG/HrpX regulon (Buttner & Bonas, 2010; Teper et al., 2021). The expression and activity of HrpG and HrpX requires regulatory factors, such as TRs (Pandey et al., 2016; Rashid et al., 2016; Zhou et al., 2017), and TCSs (Li et al., 2014; Lin et al., 2022; Subramoni et al., 2012). In this study, we found a new TR, Sar, that directly binds to the promoter region of *hrpG* and *hrpX* for their expression (Figure 5). In the promoter region of *hrpX*, the Sar binding site (ATCGTGTGCGCCAGC) is downstream of the HrpG binding site (ATTCCATT) and these two binding sites are separated by only 16 base pairs (Figure 5c). Sar protein may have a physical interaction with HrpG protein (data not shown). We think that HrpX is the direct executor, while Sar is a small switch upstream of HrpX, but not a critical switch. We found that Sar protein is conserved in *Xanthomonas* spp. (Zheng et al., 2021) and other genera (Figure S6). This study advances our understanding of the fine-tuned regulation of pathogenicity regulatory networks in *Xanthomonas*. Future work should figure out the relationship of the different regulators that regulate the HrpG/HrpX regulon.

4 | EXPERIMENTAL PROCEDURES

4.1 | Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table S1. *E. coli* strains were grown aerobically at 37°C in Luria-Bertani (LB) medium. Xoo strains were routinely cultivated at 28°C in NB medium (polypeptone 5 g/L, yeast extract 1 g/L, sucrose 20 g/L, beef extract 3 g/L, pH 7.0) or minimal XOM2 medium (D-xylose 1.8 g/L, methionine 0.099 g/L, sodium glutamate 1.87 g/L, KH_2PO_4 2 g/L, $MnSO_4$ 0.006 g/L, $MgCl_2$ 0.2 g/L, pH 6.5–7.0). If necessary, antibiotics were added at the following concentrations: Xoo, kanamycin (50 μg/ml), spectinomycin (100 μg/ml); for *X. campestris* pv. *campestris*, rifampicin (25 μg/ml); for *E. coli*, spectinomycin (100 μg/ml), kanamycin (50 μg/ml), chloramphenicol (34 μg/ml), streptomycin (8 μg/ml), and tetracycline (12.5 μg/ml).

4.2 | Bacterial genetic manipulation

According to previous studies, the suicide vector pK18mobSacB was used to construct the in-frame deletion mutants based on the homologous double-crossover method (Zheng et al., 2018). pHM1, pET-28a, pTRG, or pBXcmT were used to construct recombinant vectors for genetic complementation or overexpression of the genes, which were then electroporated into the corresponding bacterial strain. All primer sequences used in this study are listed in Table S2.

4.3 | Virulence assay

The Xoo strains were cultured in NB medium overnight and washed twice by ddH_2O, then the OD_{600} was adjusted to 0.8. The 4- to 5-week-old rice plants (susceptible rice cultivar IR24) were inoculated with different Xoo strains by the leaf-clipping method (Yang & Bogdanove, 2013). Disease symptoms were measured 14 days post-inoculation. The experiments were repeated at least three times.

4.4 | RT-qPCR

Xoo strains were cultured in XOM2 medium to the logarithmic phase (OD_{600} at 0.5). According to the manufacturer's protocol, the RNA of different cell pellets was extracted using an EasyPure RNA Kit (TransGen Biotech). Afterwards, the crude RNA was digested with DNase I (RNase-free) followed by a PCR procedure to ensure that the RNA samples were DNA-free. Then, the purified RNA was reverse transcribed to cDNA using a cDNA synthesis kit (Takara Bio) based on the manufacturer's protocol. Relative quantification of gene expression was performed using the 16S rRNA gene as an internal control.

4.5 | Bacterial one-hybrid

The open reading frame of *sar* was used to construct the pTRG-*sar* plasmid for the bacterial one-hybrid assay. Then, pTRG-*sar* and 396 pBXcmT-promoter plasmids were co-transformed into *E. coli* XR (XL1-Blue MRF'). Subsequently, 396 clones were cultured in both nonselective and selective screening media to detect the interaction of Sar with the 396 promoters. The promoter IDs are listed in Table S1.

4.6 | EMSA

The *sar* open reading frame was cloned into the pET-28a vector to express hexahistidine-tagged Sar. According to the manufacturer's instructions, Sar expressed in *E. coli* Rosetta (DE3) was purified using a Ni-NTA affinity column (Beijing CoWin Biotech). The purified protein was then dialysed in 20 mM Tris–HCl (pH 8.0) buffer supplemented with 5% glycerol and 150 mM NaCl to remove the residual imidazole. The promoter DNA probes were amplified and purified with a Gel Extraction Kit (Omega Bio-tek). FAM-labelled DNA probes were incubated with Sar protein in an improved EMSA buffer [20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM dithiothreitol (DTT), 0.1 mg of bovine serum albumin per ml and 5% glycerol] at 28°C for 45 min (Huang et al., 2008). For the competitive assay, different amounts of unlabelled probe were added to the EMSA system. Samples were then loaded on a 5% polyacrylamide gel prepared and run in 0.5× Tris-Borate buffer at 120 V for 45 min. The positions of the labelled probes in the polyacrylamide gel were visualized by a Typhoon Scanner (GE Healthcare). For the unlabelled EMSA, the gel was stained with ethidium bromide after electrophoresis, followed by image display under ultraviolet light.

4.7 | GUS assay

The promoters of *raxST* and *raxX* were cloned in pHG3 plasmid (Zou et al., 2021) and transformed into WT strain $PXO99^A$ and the Δ*sar* mutant strain. The activity of β-glucuronidase (GUS) was measured as described (Jefferson et al., 1986). Briefly, Xoo strains were cultured in XOM2 medium until the OD_{600} was 0.5. Cell pellets were collected and sonicated to obtain the supernatant. The absorbance of *p*-nitrophenol was measured at 415 nm using 4-nitrophenyl β-D-glucopyranoside (PNPG) as a substrate.

4.8 | Interbacterial competition assay

Xoo strains ($PXO99^A$, Δ*sar*, Δ*hcp1*, Δ*hcp2*, Δ*hcp1*Δ*hcp2*), Xcc 8004 (resistant to rifampicin), and *E. coli* XL1-Blue (resistant to kanamycin) were cultivated to log-phase and resuspended to OD_{600} = 0.8. Xoo cells were then mixed with Xcc 8004 or *E. coli* XL1-Blue in a ratio of 10:1. A total of 5 μl of the above mixtures was spotted on NB agar plates and incubated at 28°C for 48 h. The spots were excised,

suspended in sterile water, and 5-μl serial 10-fold dilutions were spotted on rifampicin NB agar plates and incubated at 28°C for 60h or on kanamycin LB agar plates and incubated at 37°C for 48h.

4.9 | Western blot

Western blot was performed using standard protocols with anti-His mouse monoclonal antibodies (Proteintech) and a goat anti-mouse antibody conjugated with horseradish peroxidase (HRP) secondary antibody (Proteintech), and visualized by a chemiluminescent detector system using Pierce enhanced chemiluminescence (Thermo Fisher Scientific) as the HRP substrate.

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DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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